THE FORMATION OF CYTOCHROME P-450 METABOLIC INTERMEDIATE COMPLEXES IN ISOLATED HEPATOCYTES

by

Stephen Michael Roberts

A dissertation submitted to the faculty of the University of Utah in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

Department of Pharmacology

University of Utah

December 1977

THE UNIVERSITY OF UTAH GRADUATE SCHOOL

SUPERVISORY COMMITTEE APPROVAL

of a dissertation submitted by

Stephen Michael Roberts

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10/14/77
Date

R. Franklin, Ph.D.
Chairman, Supervisory Committee

I have read this dissertation and have found it to be of satisfactory quality for a doctoral degree.

Date

W. M.D., Ph.D.
Member, Supervisory Committee

I have read this dissertation and have found it to be of satisfactory quality for a

Date

Ph.D.
Member, Supervisory Committee

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Sweat, Ph.D.
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[redacted] 77
Date

[redacted]
Michael R. Franklin, Ph.D.
Member, Supervisory Committee

Approved for the Major Department

[redacted] M. [redacted]
Chairman/Dean

Approved for the Graduate Council

[redacted]

ABSTRACT

This thesis examines the formation of cytochrome P-450 metabolic intermediate (MI) complexes in isolated hepatocytes. The validity of the model was established, and comparisons were made between MI complex formation in hepatocytes and microsomal suspensions derived from untreated and phenobarbital-pretreated rats. The rates of MI complex formation, the presence of the substrate optimum effect for certain substrates, and the stability of the MI complexes were compared. Information from these comparisons was used in the study of possible mechanisms for inconsistent observations on MI complex formation between microsomes and isolated perfused livers and whole animals.

The relationship between substrate concentration and rate of reaction was established for N-hydroxyamphetamine, SKF 525-A, and norbenzphetamine in hepatocytes from phenobarbital-pretreated rats, as well as for N-hydroxyamphetamine and norbenzphetamine in hepatocytes from untreated rats. These relationships will provide a basis for comparison in future studies of MI complex formation in isolated perfused livers. The stability of MI complexes formed in vitro

in microsomes was examined, and it was determined that the MI complexes are unstable under certain conditions. The rates of decay are influenced by temperature, and the degree of influence varies depending on the substrate from which the MI complex is formed.

Hepatocytes isolated from drug-treated rats were found to contain similar amounts of MI complex as microsomes from identically treated animals.

The implications of the results on possible mechanisms underlying the discrepant *in vitro* and *in vivo* observations are discussed.

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INTRODUCTION

The ability of a drug to influence its own rate of metabolism and the metabolism of other drugs comprises a substantial aspect of the study of drug interactions. As the enzyme cytochrome P-450 is responsible for the mixed-function oxidation of a wide variety of compounds, drugs which inactivate this enzyme have the potential for modifying the effect of concurrently administered drugs.

Many compounds have been shown to form complexes with cytochrome P-450 characterized by an absorbance maximum in the Soret region at or near 455 nm (Franklin, 1977). Mixed-function oxidation of the compound is required for this complex formation, and evidence indicates that it is a metabolic intermediate (MI) rather than the parent compound or product which binds with cytochrome P-450. These complexes render the enzyme inactive and may alter the rate of metabolism of other substrates undergoing mixed-function oxidation (Franklin, 1972; Schenkman et al., 1972; Werringloer and Estabrook, 1973a; Franklin, 1974a). The formation of cytochrome P-450 MI complexes from amines has been demonstrated in vitro with hepatic

microsomes from the mouse, rat, guinea pig, rabbit, dog (James and Franklin, 1975a), monkey, and man (Roberts and Franklin, unpublished results).

Implicated in the formation of these complexes are drugs representing several pharmacologic categories, including central nervous system stimulants (amphetamine, methamphetamine, benzphetamine, etc.) and their congeners (l-amphetamine, norbenzphetamine, etc.), analgesics (propoxyphene, methadone, l-α-acetylmethadol), antihistamines (diphenhydramine), anticholinergics (adiphenine, benactyzine), sympathomimetic vasoconstrictors (propylhexedrine, cyclopentamine), and tricyclic antidepressants (desipramine, nortriptyline) (Franklin, 1973, 1974a, 1974b; Werringloer and Estabrook, 1973a, 1973b; Buening and Franklin, 1974; James and Franklin, 1975b; Roberts and Franklin, 1976; Franklin and Roberts, 1977).

The formation of these complexes is closely related to hepatic induction (Franklin, 1974c). Induction with phenobarbital, but not 3-methylcholanthrene, appears to induce a subpopulation of cytochrome P-450 susceptible to MI complex formation, as phenobarbital induction increases the specific activity of nearly all such MI complex-forming reactions. For example, a three-fold increase in cytochrome P-450 concentration may result in a 30-fold increase in the rate of MI complex formation (Franklin, 1974c).

Experiments attempting to detect the formation of MI complexes in whole animals, or under the more controlled conditions of the isolated perfused liver, have yielded results dissimilar to those obtained with microsomes *in vitro* (Table 1). While comparable amounts of MI complex from SKF 525-A were found in all three model systems, substantial quantitative differences existed between whole animals and isolated perfused livers on the one hand, and microsomes on the other for propoxyphene, N-hydroxyamphetamine, and norbenzphetamine.

There are at least two explanations for this apparent discrepancy between *in vitro* and *in vivo* observations: 1) less MI complex is formed *in situ*, or, 2) comparable amounts of complex are formed but are disrupted or decay before they can be measured. In extrapolating animal data and human *in vitro* data to clinical situations in man, it is very important to distinguish between these two possibilities. Both the amount of cytochrome P-450 complexed and its potential mixed-function oxidation activity will determine the level of inhibition of drug metabolism created by the formation of MI complexes. If significantly less MI complex is, in fact, formed *in vivo* as compared to similar tissue *in vitro*, then the use of *in vitro* data may give rise to an overestimation of

TABLE 1

Maximum Amount of MI Complex Observed

Substrate	Percent Cytochrome P-450 as MI Complex		
	Microsomes	Isolated Perfused Livers[a]	Whole Animals
SKF 525-A	46[a]	21	41[c]
Propoxyphene	59[a]	10	16[d]
N-hydroxyamphetamine	80[b]	4	4[a]
Norbenzphetamine	64[a]	16	0

[a]Franklin, unpublished results

[b]Franklin, 1974d

[c]Buening and Franklin, 1976

[d]Roberts and Franklin, 1976

the inhibitory potential of a particular complex-forming drug. If, on the other hand, the techniques available to measure *in vivo* MI complex formation result in complex disruption or decay during sample preparation, artificially low values of MI complex formation would be obtained. The use of these *in vivo* results could create a substantial underestimation of inhibitory potential. Postulated mechanisms for the two alternatives are discussed below.

Less MI Complex is Formed In Situ

The most attractive explanation for why an *in vivo* system would form low amounts of MI complex involves the substrate optimum phenomenon. In microsomes, the initial rate of MI complex formation (which is the maximum rate) is dependent upon, among other things, the concentration of substrate. The rate of reaction increases with substrate concentration until an optimum level is reached, illustrated for norbenzphetamine in Figure 1. Concentrations of substrate beyond this optimum yield progressively slower initial rates of reaction and lower extents of formation (Franklin, 1974a; Buening and Franklin, 1974). The optimum concentration value depends upon the substrate and appears to be independent of enzyme (cytochrome P-450) concentration (Franklin, 1974c).

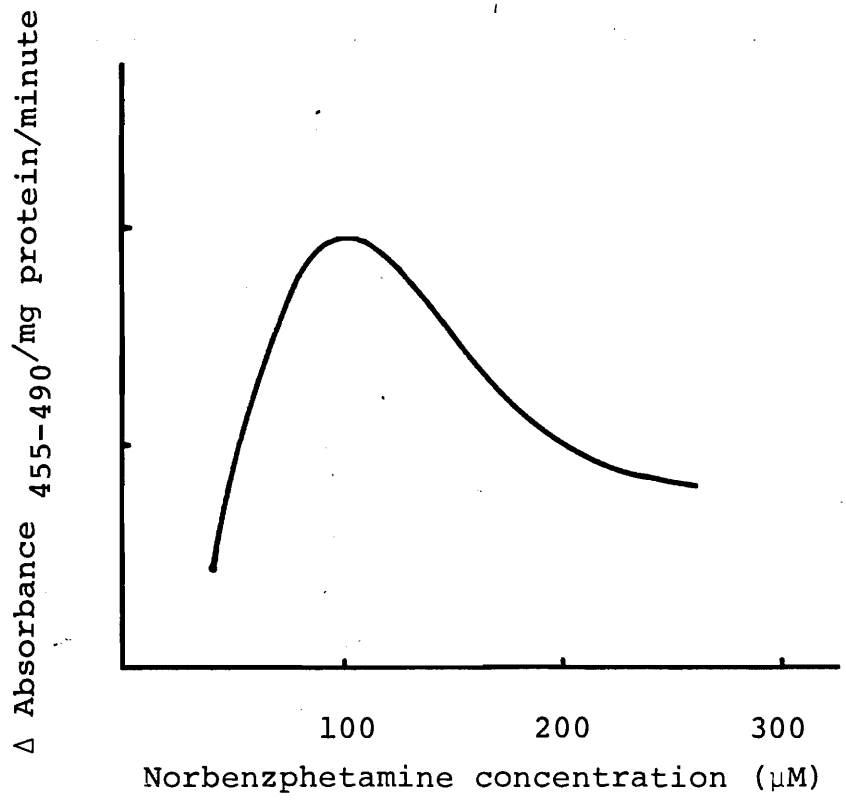


Figure 1. Initial Rate of MI Complex Formation From Norbenzphetamine in Hepatic Microsomes From Phenobarbital-Pretreated Rats

A role for product inhibition in this phenomenon is untenable as the initial rates are affected. Product inhibition may explain the observation that super-optimal substrate concentrations do not result in substantial complex formation despite the fact that with time and sufficient NADPH and oxygen, they must be metabolized into the optimum MI complex-forming concentration range. Also, super-optimal substrate concentrations could alter microsomal membrane structure resulting in lower rates and extents of MI complex formation.

The substrate optimum effect has not been demonstrated in isolated perfused livers or whole animals, and, indeed, it would be technically very difficult to do so. If this effect was not unique to microsomes, however, low levels of MI complex could, perhaps, be explained by inappropriate substrate concentrations presented to the cytochrome P-450 in experiments with these model systems (Table 2).

MI Complex is Disrupted or Decays Before Measurement

Previous observations (Franklin, unpublished results) have indicated that MI complexes may be destroyed during microsome preparation. Microsomes containing MI complex formed *in vitro*, when resubjected to the conditions of their preparation (homogenization,

TABLE 2

Concentration Ranges Employed in Isolated Perfused Liver Studies[a]

Substrate	Concentration Range[b]
N-hydroxyamphetamine	47 - 232 μM
Norbenzphetamine	37 - 185
SKF 525-A	26 - 135

[a]Franklin, unpublished results.

[b]Total drug concentration (bound and free) in perfusate consisting of Tyrodes solution with 4% albumin.

centrifugation, etc.), were found to have lost measureable amounts of complex. Since microsome production is an obligatory intermediate step in the assaying of the MI complex formed in whole animals and in isolated perfused livers, results from these systems may not accurately represent the amount of complex *in situ*.

The most direct approach to these questions required the study of MI complex formation in a fourth model system, the isolated hepatocyte suspension. The isolated hepatocyte suspension would allow for the examination of the substrate optimum effect and stability of MI complexes from different substrates in the intact parenchymal cell and provide a workable intermediate model between microsomal suspensions and isolated perfused livers.

EXPERIMENTAL APPROACH

An isolated hepatocyte suspension model system was developed for the rat from descriptions in the literature in an attempt to reconcile disparate observations on *in vivo* and *in vitro* MI complex formation. This model system, like whole animal studies and the isolated perfused liver, contains the complex mechanisms of the intact liver cell. However, hepatocytes are sufficiently translucent to afford direct spectrophotometric examination and need not undergo microsome preparation as with other model systems (Figure 2).

Isolated hepatocyte suspensions were employed to study the *in vitro* and *in situ* formation of MI complexes. The following questions were investigated:

1. Do MI complexes form in the intact parenchymal cell?
2. Do isolated parenchymal cells show the substrate optimum phenomenon?
3. How does complex formation in isolated parenchymal cells compare with microsomes?
4. How does complex stability in isolated parenchymal cells compare with microsomes?

5. Can complex stability concerns with *in vitro* assays of *in vivo* formation be overcome by using hepatocytes isolated from drug-treated animals, eliminating the need for microsome preparation?

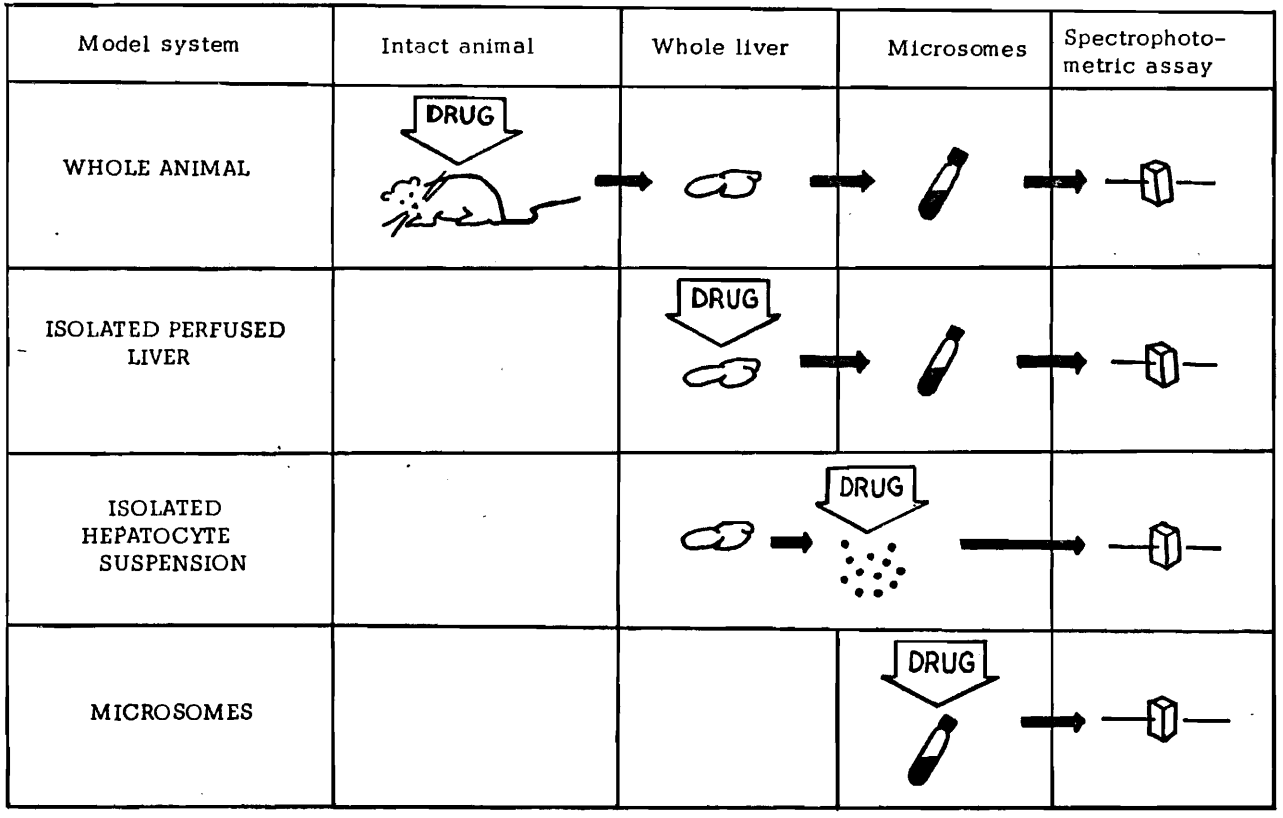


Figure 2. Preparatory Steps in the Assay for Metabolic-Intermediate Complexes

METHODS

Isolated Hepatocyte Suspension Preparation

Introduction

To understand the process of cell dispersion, it is important to examine the arrangement of hepatocytes in liver tissue and the fine structure of the parenchymal cell membrane (Figure 3).

Each parenchymal cell is surrounded by extracellular space of three types: the space of Disse which separates the parenchymal cell from the sinusoid, a specialized capillary; the lumen of the bile canaliculus; and the space lying between neighboring parenchymal cells. Microvilli line that portion of the membrane exposed to both the space of Disse and bile canaliculus.

Structure is provided by collagen fibrils, and cells are held together both by attachment at specialized membrane segments and by numerous "stud-like" projections from one cell into another. Three types of specialized membrane segments have been identified: tight junctions, intermediate (or gap) junctions, and desmosomes (Farquhar and Palade, 1963).

Figure 3. Diagramatic Sketch of Liver Tissue Ultrastructure. Key to Symbols.

bc = bile canaliculus

d = desmosome

ds = Disse space

ij = intermediate junction (or gap junction)

kc = Kupffer cell

m = mitochondrion

mv = microvilli

n = nucleus

pc = parenchymal cell

s = sinusoid

sp = studlike projection

tj = tight junction

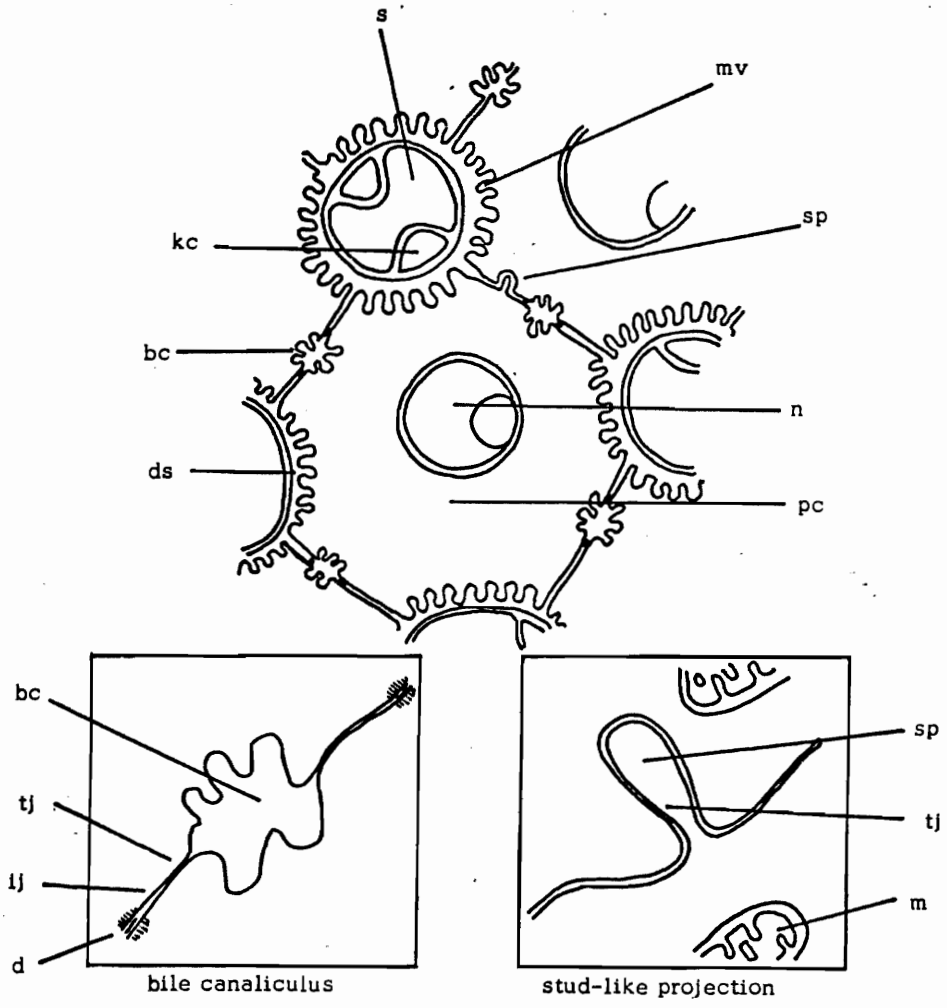


Figure 3. Dragramatic Sketch of Liver Tissue Ultra-structure

The tight junction (zonula occludens) is the result of a fusion of the membranes of adjacent cells. In combination with an intermediate junction and desmosome (termed "junctional complex"), it closes the margins of the bile canaliculus, sealing it from other extracellular space. In addition, tight junctions of varying lengths may be found at random locations between cells, including the stud-like projections.

In contrast to the tight junctions, the intermediate junction (zonula adhaerens) has an extracellular space of about 200 Å and is filled with a low density material. The desmosome (macula adhaerens) also has an extracellular space of about 240 Å which contains a central disc of dense material.

In order to isolate the parenchymal cells, junctions of all three types, as well as the collagen fibrils, must be cleaved. No single step yet developed can accomplish this without irreparable cell damage. Thus, a series of steps have evolved to allow separation with a minimum of cell damage.

Current methodology for hepatocyte separation involves sequential steps of calcium removal, enzyme digestion, and mechanical dispersion to achieve high yields of viable cells. Deletion of any of these three steps will result in low yields or cells of poor quality.

Mechanical Dispersion

The first attempts to isolate hepatocytes utilized purely mechanical means, including forcing the tissue through cheesecloth (Schneider and Potter, 1943), shaking the tissue with glass beads (Bucher et al., 1951; St. Aubin and Bucher, 1952), mincing with a tissue press (Kaltenbach, 1952), and homogenization with a Potter-Elvehjem grinder (Palade and Claude, 1949). While the products of these methods were uniformly poor, some mild form of mechanical force remains necessary, as neither calcium removal nor enzyme digestion are capable of breaking the tight and intermediate (or gap) junctions (Berry and Friend, 1969).

The mechanical step is generally employed last and usually consists of some form of shaking or aggitation of the enzyme-treated suspension.

Calcium Removal

Responding to earlier reports of a possible role for calcium in intercellular binding (Gray, 1926; Zeidman, 1947; and Northcraft, 1951), Anderson (1953) employed a series of divalent cation-binding compounds (citrate, EDTA, etc.) to facilitate mechanical dispersion of the cells with good result. Yields up to 73% were obtained, in sharp contrast to the 5-10% yields of previous investigators relying solely on mechanical methods.

Branster and Morton (1957) reported results suggesting that the use of chelators was not required and that perfusing with only a calcium-free buffer resulted in the removal of sufficient calcium to facilitate separation. Seglen (1972, 1973a) studied in detail the roles of ion removal and chelators in cell separation and explained that although the use of EGTA increased cell dispersion, the use of chelators was probably not indicated in view of their potential toxicity to the cell (Kalant and Miyata, 1963).

Studies by Hays et al. (1965) with toad bladder indicated that the removal of calcium resulted in the cleavage of desmosomes. Other investigators (Berry and Friend, 1969; Drochmans et al., 1975) have confirmed the same for parenchymal cells. Experiments performed with pancreatic tissue suggest that the central disc of the desmosome has a Ca^{+2}-dependent factor, and the loss of adherence in the absence of calcium causes the desmosome to cleave (Amsterdam and Jamieson, 1974).

Calcium removal is typically accomplished as a first-step perfusion with a calcium-free buffer, with or without a chelator.

Enzyme Digestion

The first reported use of a collagenase-hyaluronidase mixture for parenchymal cell dispersion was

performed by Howard et al. (1967). Livers were flushed with an enzyme mixture, cut into slices, and then incubated with the enzyme solution. While yields obtained from this procedure were quite poor, 3-5%, evidence was presented suggesting that the quality of the cells was very good. By perfusing the liver with a collagenase-hyaluronidase combination for about 20 minutes, Berry and Friend (1969) noted that yields up to 60% could be obtained.

While the value of collagenase perfusion in cell separation is now well-established, the inclusion of hyaluronidase has recently been questioned (Seglen, 1973b; Berry, 1976). Experience in our laboratory would verify the observation that hyaluronidase inclusion does little to increase yields.

The use of collagenase is complicated somewhat by its requirement for calcium (Gallop et al., 1957). While calcium removal and the subsequent cleavage of desmosomes is essential for obtaining a good preparation (Berry, 1976), some calcium must be present for collagenase activity. The most practical solution to this dilemma involves two sequential perfusions, one to remove calcium, the other to dissolve collagen. Some investigators, particularly those using chelators for calcium removal, find it necessary to add calcium during

the enzyme perfusion (Seglen, 1972, 1973a). Others have found this addition unnecessary, and experience in our laboratory and others (Berry, 1976; F. Sweat, personal communication) indicates that calcium addition during the enzyme perfusion results in lower yields.

The ability of collagenase to function in a calcium-free buffer is something of a paradox. However, commercial collagenase contains tightly bound calcium, apparently sufficient for activity. It has even been suggested that the activity of commercial collagenase may be related to its calcium content (Berry, 1976).

Perfusion Apparatus

Modifications were made from the apparatus described by Miller et al. (1951) to make the perfusion system more suitable for cell isolation (Figure 4). The modified system used two independent perfusate circuits originating from the reservoir. One circuit pumped perfusate directly through the liver, the other was for oxygenation of the perfusate and pH monitoring. A third circuit delivered a humidified 95% oxygen/5% carbon dioxide mixture to the glass lung and the reservoir. These circuits are described below.

Perfusion Circuit

Perfusate from the reservoir was pumped directly through the liver and either eliminated from the system

Figure 4. Perfusion Apparatus. Key to Symbols.

1. glass lung
2. humidifiers
3. water trap and valve
4. pH electrode
5. overflow for glass lung
6. funnel for adding perfusate
7. exit cannula
8. induction cannula
9. pump*
10. vacuum line for recovering perfusate
11. sump
12. perfusate reservoir

*single pump with two heads may be used.

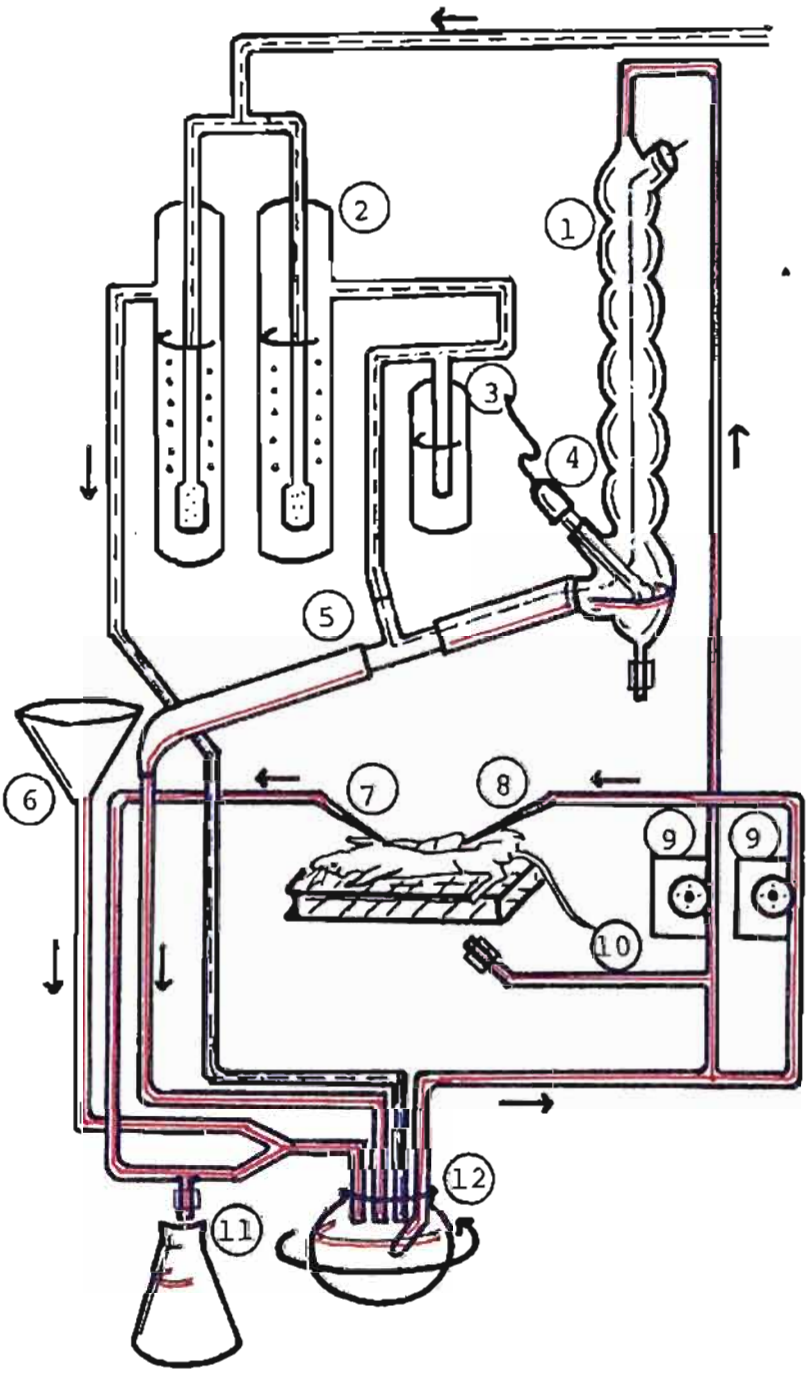


Figure 4. Perfusion Apparatus

(during the first perfusion) or returned to the reservoir (during the second, enzyme perfusion). The rate of perfusion was controlled directly by the speed of the calibrated pump.

Oxygenation Circuit

Perfusate was pumped from the reservoir to the top of the glass lung, where its sheet-like flow down the glass walls facilitated the dissolution of oxygen. A small reservoir at the bottom of the lung collected gassed perfusate, and it was here that the pH was monitored. Overflow from this reservoir was directed, against the flow of gas, back to the main reservoir.

The main reservoir was itself gassed with the 95% O_2/5% CO_2 mixture, exchange being facilitated by rapidly rotating the reservoir flask with its axis at about a 60° angle (not shown in figure).

Prior to entering either the lung or reservoir, gas was humidified by passing through distilled water. Evaporation of the perfusate was thereby minimized.

A funnel leading into the main reservoir was incorporated to simplify introduction of perfusate into the system. A "vacuum line" branching off of the reservoir-to-lung line below the pump was also added. If the preparation leaked perfusate, it could be recovered using this line.

The entire apparatus was enclosed in a temperature-controlled (37°) plexiglass incubator (Precision Scientific Co., Chicago, Illinois). Specialized glassware was obtained from MRA, Inc. (Boston, Massachusetts).

Surgery

The rat was anesthetized with ether, and the abdominal cavity opened with incisions along the midline and laterally beneath the ribs. The intestinal bulk was gently lifted from the cavity and placed to the side exposing the portal vein and vena cava. Three hundred units of heparin were injected into the inferior vena cava and allowed to circulate for about two minutes. The portal vein was then isolated and cannulated with the induction cannula. The inferior vena cava was ligated at a position between the hepatic and right renal veins. The thoracic cavity was next opened, and the inferior vena cava cannulated just about the diaphragm with the exit cannula. Finally, 10-20 ml of normal saline were injected through the system via the induction cannula to check for leaks and cannula misplacement. The rat with the liver isolated *in situ* was then ready for perfusion.

Liver Perfusion

Two sequential perfusions were performed, both using a calcium-free modified Hanks balanced salt solution (MHBSS) (Table 3). The purpose of the first perfusion was to eliminate blood and calcium from the liver. One hundred to 150 ml of buffer were passed through the liver and allowed to flow into a collection sump. Perfusion time was about 5 minutes.

When the buffer in the reservoir was depleted, it was replaced, this time with medium containing 80 mg% collagenase (Type I, Sigma Chemical Co., St. Louis, Missouri). Perfusion was resumed at a rate of about 20 ml/min. The valve to the sump was closed, and the perfusate was allowed to return to the reservoir. The enzyme perfusion took about 20 minutes and the pH remained between 7.5 and 7.6. Care was taken to ensure that no air bubbles were allowed to pass through the liver.

After about 15 minutes of enzyme perfusion, the liver became quite soft. Perfusion was halted at the appearance of obvious signs of liver disintegration.

Cell Washing

The liver, as it was removed from the animal carcass, contained primarily connective tissue, chains of parenchymal cells, and reticuloendothelial (RE or

TABLE 3

Modified Hanks Balanced Salt Solution
(MHBSS)

Component[a]	Concentration (mg/l)
NaCl	8000
KCl	400
$CaCl_2$[b]	440
$Na_2HPO_4 \cdot 2H_2O$	60
KH_2PO_4	60
Glucose	1000
$NaHCO_3$	2100
N-2-hydroxyethylpiperazine N'-2-ethanesulfonic acid (HEPES)	5950

[a]All components were obtained from J.T. Baker Chemical Co., Phillipsberg, New Jersey, except for HEPES which was obtained from Sigma Chemical Co., St. Louis, Missouri.

[b]Deleted in calcium-free modified Hanks BSS.

Kupffer) cells, with lesser amounts of cellular debris and trace numbers of erythrocytes. The first step in the recovery of the parenchymal cells was to split the tight and intermediate junctions freeing the individual cells.

To accomplish this, the liver mass and about 50 ml of fresh enzyme-perfusate were rotated rapidly in a vessel similar to that used as the perfusion reservoir. Oxygenation of the cells was maintained by blowing 95% O_2/5% CO_2 into the mouth of the vessel. In addition to cleaving the tight junctions, this mechanical step disrupted the damaged cells, making their separation from the viable, intact cells easier.

The suspension was filtered three times through nylon mesh to remove connective tissue.

The suspension was centrifuged at 40 x g for three minutes, and the supernatant containing the enzyme, erthyrocytes, and the numerous but relatively small reticuloendothelial cells was removed. The cells were gently resuspended with calcium-free MHBSS and recentrifuged. After removal of the supernatant, the resuspension in calcium-free MHBSS and centrifugation were repeated. Again, the supernatant was removed, and the cells were finally suspended in complete MHBSS to a concentration of about 6×10^6 cells/ml.

Cell concentration was determined by counting in a hemacytometer.

The washing successfully removed virtually all of the erythrocytes and reticuloendothelial cells, leaving a single-cell suspension of parenchymal cells. As this study involved only mixed-function oxidation reactions (MI complex formation) and the RE cells are devoid of cytochrome P-450 (Hupka and Karler, 1973), only the parenchymal cells were of interest. For the purposes of this study, the terms "cells" and "hepatocytes" will refer to hepatic parenchymal cells.

Viability Testing

Introduction

The quality of cells isolated can vary considerably, and it is useful to have some objective measure of cell viability so that standards for cell quality may be set. Unfortunately, no single test can adequately index the structural and biochemical integrity of a cell. While investigators may focus on the functional state of a particular cell process to be studied, it is often desirable to have come criteria for evaluating the general viability of the cell preparation.

General viability tests are, for the most part, limited to assays designed to gauge the intactness of of the plasma membrane. This structure is particularly vulnerable to injury during the isolation process, resulting in possible leakage of cytoplasmic contents and the loss of membrane potential. Examples of such general viability tests include trypan blue dye exclusion, lactate dehydrogenase leakage, membrane potential measurements, intracellular Na^+ and K^+ concentrations, and stimulation of cell respiration by added succinate. Other general viability tests not directly related to plasma membrane integrity include cell respiration rate, uridine incorporation, and ATP/ADP ratio.

As yet, no general viability test, or series of tests, can be considered standard, although trypan blue dye exclusion is probably the most common test employed. The confusion generated by the absence of a standard test is made worse by the lack of standard procedures for many of these tests.

While a cell preparation is best characterized by several different tests, there are practical limitations to the number of tests that can be performed. Cell suspensions deteriorate rapidly, even when stored under optimal conditions. The viability testing must,

therefore, be brief in order to allow enough time for the intended experiments.

Three viability tests were used routinely for this study, trypan blue dye exclusion (TBDE), lactate dehydrogenase activity (LDHA), and stimulation of cell respiration by added succinate (SCRS). A fourth test not described previously, NADPH-norbenzphetamine stimulation of cell respiration, was developed but not used routinely. These tests are described in detail below.

Trypan Blue Dye Exclusion (TBDE)

TBDE is a very quick and easy test to perform. At neutral pH, cells which take up the stain are regarded as being severely and irreversibly damaged (Baur et al., 1975) (Plate 1). Permeability of the plasma membrane to trypan blue has been associated with a decreased uptake of amino acids (Dickson, 1970) and leakage of cytoplasmic lactate dehydrogenase (Baur et al., 1975).

TBDE is not a particularly sensitive test of cell viability, and cells which do not take up the stain may nonetheless show signs of obvious structural damage (Plate 2) as well as other signs of injury (e.g., low membrane potential, uridine incorporation, and ATP/ADP ratio) (Baur et al., 1975).

PLATE 1. The cell on the left is an intact, viable parenchymal cell in the presence of 0.06% trypan blue. It does not show the same dark staining of the nuclei as the pair of cells on the upper right. Note that the upper stained cell appears to be binucleate, a situation not uncommon among parenchymal cells.

PLATE 2. Both cells have been exposed to 0.06% trypan blue. The cell on the upper left has taken up the stain and is considered nonviable by trypan blue dye exclusion (TBDE) standards. The cell on the lower right shows the balloon-like projections which appear when the cells are kept at 25° or 30°. Despite the presence of these projections, this cell has not taken up appreciable stain and would be considered viable by TBDE standards.

Both photomicrographs were taken at 375 X magnification (40 X objective, 15 X ocular, 50 mm bellows extension) with a Zeiss phase contrast microscope. Kodak KPA 135 film, ASA 40, was used.

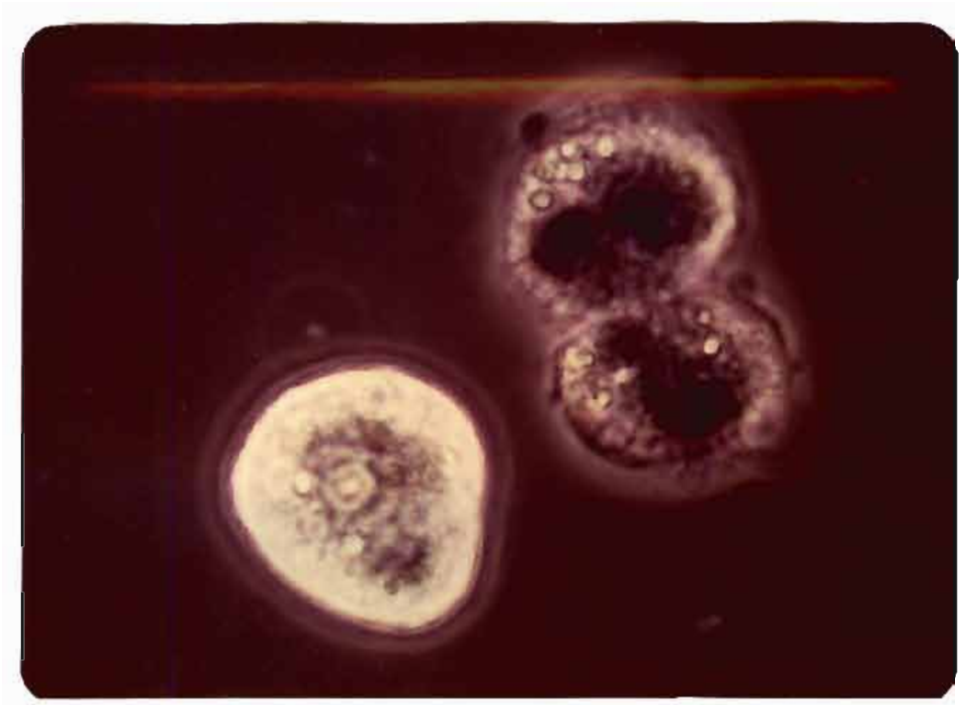


PLATE 1

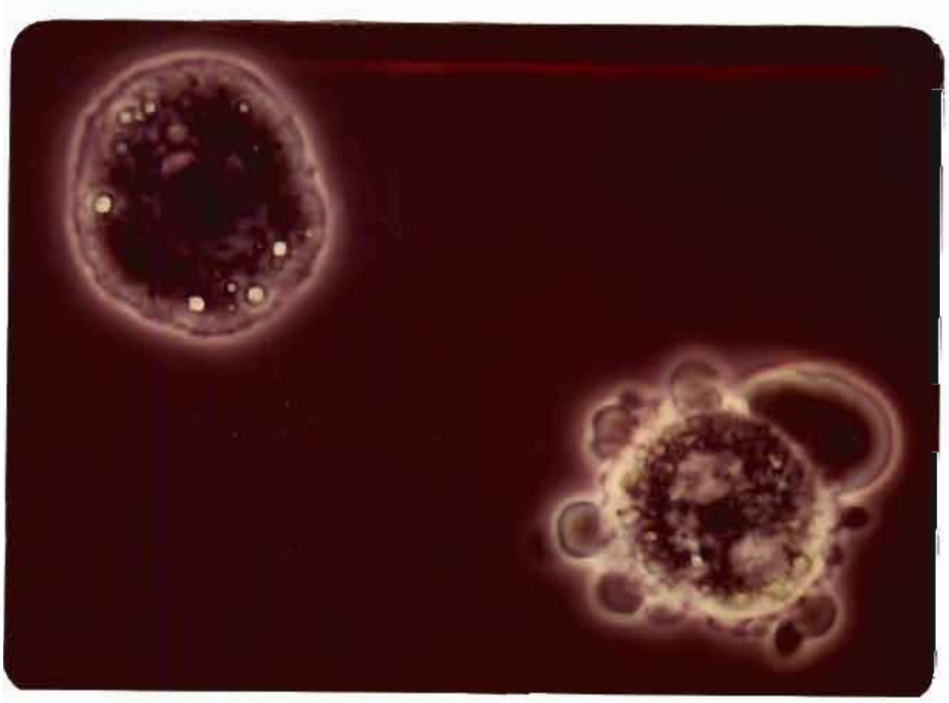


PLATE 2

Despite its deficiencies, TBDE is presently the most common test against which the preparations of different investigators are compared.

Method. The stain was prepared by dissolving solid trypan blue (MCB, Norwood, Ohio) in normal saline to a concentration of 0.12% and filtering through a 0.45 μ mesh (Millipore Corp., Bedford, Massachusetts).

Equal volumes of stain and cell suspension were mixed and placed in a hemacytometer for counting (final dye concentration of 0.06%). After examining approximately 200 cells, the percent taking up the stain was determined. The procedure was repeated, and the two values averaged. Incubation time of the stain with the cells before counting was usually less than one minute.

Unless otherwise noted, cell preparations showing more than 10% of cells taking up the stain were rejected.

Lactate Dehydrogenase Activity (LDHA)

Lactate dehydrogenase is a cytoplasmic enzyme, and the presence of this protein outside the cell is indicative of substantial and probably irreversible

cell damage. As such, LDHA, like TBDE, is not a "sensitive" test of general viability and reflects gross, rather than subtle, abnormalities in the cell population. The assay is not, however, functionally interchangeable with TBDE, and data suggest there are circumstances in which one test will indicate damage while the other does not (Table 4).

The assay for LDHA is based on the following reaction:

$$\text{pyruvate} + \text{NADH} \xrightarrow{\text{lactate dehydrogenase}} \text{NAD}^{+} + \text{lactate}$$

NADPH may also participate in the reaction but produces a rate two orders of magnitude lower.

If pyruvate and NADH are added to a system containing LDH, the NADH will be oxidized causing a decrease in absorbance at 340 nm. The rate at which this absorbance is diminished, and NADH consumed, provides a measure of LDH activity.

Method. Some experimentation was required to find quantities of pyruvate and NADH such that the reaction would not be substrate-limited. The following procedure was established.

TABLE 4

The Effects of Magnetic Stirring on Trypan Blue Dye Exclusion and Lactate Dehydrogenase Activity of Hepatocyte Suspensions

	Time	Trypan blue dye exclusion exclusion (% excluding)	Lactate dehydrogenase activity (% 0 time sample)[a]
Unstirred	0	89	100[b]
	10	89	139
	20	80	148
Stirred	0	84	100[c]
	10	84	242
	20	84	325

[a]An increase in lactate dehydrogenase activity indicates cell damage.

[b]Lactate dehydrogenase activity of 23 nmoles NADH oxidized/10^6 cells/minute.

[c]Lactate dehydrogenase activity of 12 nmoles NADH oxidized/10^6 cells/minute.

One hundred µl of cell suspension was added to 2.8 ml of MHBSS (pH 7.4, 25°), and the absorbance measured at 340 nm in a temperature-regulated (25°) Aminco DW-2 UV/Vis spectrophotometer operating in the split beam mode. Ten µl of NADH (35 mM) were added, and the background rate of NADH oxidation, if any, was recorded. One hundred µl of pyruvate (23 mM) were then added, and the rate of LDH oxidation of NADH was measured.

In order to determine the total LDH activity, i.e., both extracellular and intracellular, a small volume of cell suspension was sonicated and the procedure repeated with 100 µl of cell suspension sonicate. From the LDH activities obtained from the whole cells and sonicate, the amount of LDH activity leaked from the cells could be determined as a percent of the total.

Cell preparations that had leaked more than 15% of the total LDH within 30 minutes after isolation were discarded. Most preparations leaked between 5 and 10% of total.

Stimulation of Cell Respiration by Exogenous Succinate (SCRS)

The addition of succinate to intact mitochondria will result in an increased rate of respiration (Hogeboom et al., 1946). The intact plasma membrane is relatively impermeable to succinate (Hems et al., 1968), and while a stimulation of cell respiration by added succinate may indicate an intact mitochondrial electron transport chain, it also indicates plasma membrane damage. The succinate molecule is relatively small compared to lactate dehydrogenase and trypan blue, and the access of added succinate to mitochondria will often preceed damage detectable by other techniques such as LDHA and TBDE. SCRS correlates very favorably with sensitive but time-consuming measurements of membrane potential and intracellular Na^+ and K^+ (Baur et al., 1975).

Method. One-half ml of cell suspension was diluted with 1.5 ml MHBSS. A Clark oxygen electrode was used to determine the oxygen tension, and the endogenous, or basal, rate of respiration was calculated. Then 50 µl of sodium succinate (Sigma Chemical Co., St. Louis, Missouri) solution was added to make a final succinate concentration of 1 mM. The respiration rate was again determined. Stimulation of respiration was expressed in terms of percent above

basal rate. Cell preparations stimulated more than 20% above the basal rate were not used for experimental purposes.

Stimulation of Norbenzphetamine Metabolism by Exogenous NADPH

The addition of a MFO substrate such as norbenzphetamine to an isolated hepatocyte suspension results in an increase in the rate of oxygen consumption. As there is evidence that MFO reactions may be NADPH-limited in the intact cell (Moldeus et al., 1974), the metabolism of norbenzphetamine and the accompanying oxygen consumption would be expected to increase with the entry into the cell of supplemental NADPH through damaged membranes. The effect of NADPH on cell respiration is not limited to a MFO mechanism, however, and an analysis of component rates had to be made (Figure 5).

The increase in respiratory rate with sequential additions of norbenzphetamine and NADPH ($R_{NADPH+NB}$) was greater than the sum of the individual increases produced by the nonspecific action of NADPH (R_{NADPH}) and the metabolism of norbenzphetamine without supplemental NADPH (R_{NB}). This suggests that NADPH does, indeed, stimulate norbenzphetamine metabolism and that if the nonspecific action of NADPH (R_{NADPH}) is

Figure 5. Stimulation of cell respiration by NADPH and norbenzphetamine. Bar A represents the endogenous rate of respiration of the cell suspension (R_{ER}); Bar B, the rate after NADPH addition ($R_{NADPH+ER}$); Bar C, the rate after norbenzphetamine addition (R_{NB+ER}); and, Bar D, the rate from sequential additions of both norbenzphetamine and NADPH ($R_{NADPH+NB+ER}$).

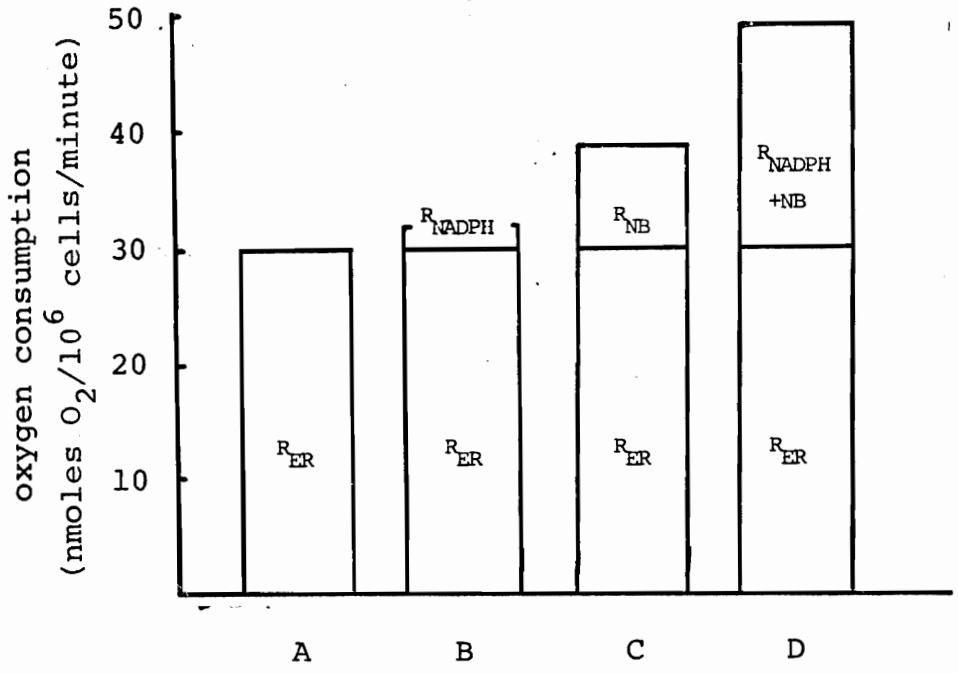


Figure 5. Stimulation of Cell Respiration by NADPH and Norbenzphetamine

substracted, the net change in norbenzphetamine metabolism can be calculated:

$$\frac{R_{NADPH+NB} - R_{NADPH}}{R_{NB}} = \frac{\text{enhanced rate}}{\text{control rate}} = \text{enhancement factor}$$

This method assumes that the endogenous rate remains unchanged with the addition of either norbenzphetamine or NADPH, and that the NADPH nonspecific rate (R_{NADPH}) remains unchanged in the presence of norbenzphetamine.

This method was compared with TBDE results for a group of samples of widely varying quality with good correlation (Figure 6).

While more time-consuming than either TBDE or SCRS, this method provides a check of the mixed-function oxidation system. It was not performed routinely in this project because of time considerations.

Method. One-half ml of cell suspension was diluted with 1.5 ml of MHBSS. The basal respiratory rate was measured with a Clark oxygen electrode. After time sufficient to establish the endogenous rate, 10 µl of 60 mM NADPH were added, and the new rate was recorded. This procedure was performed twice and the results averaged.

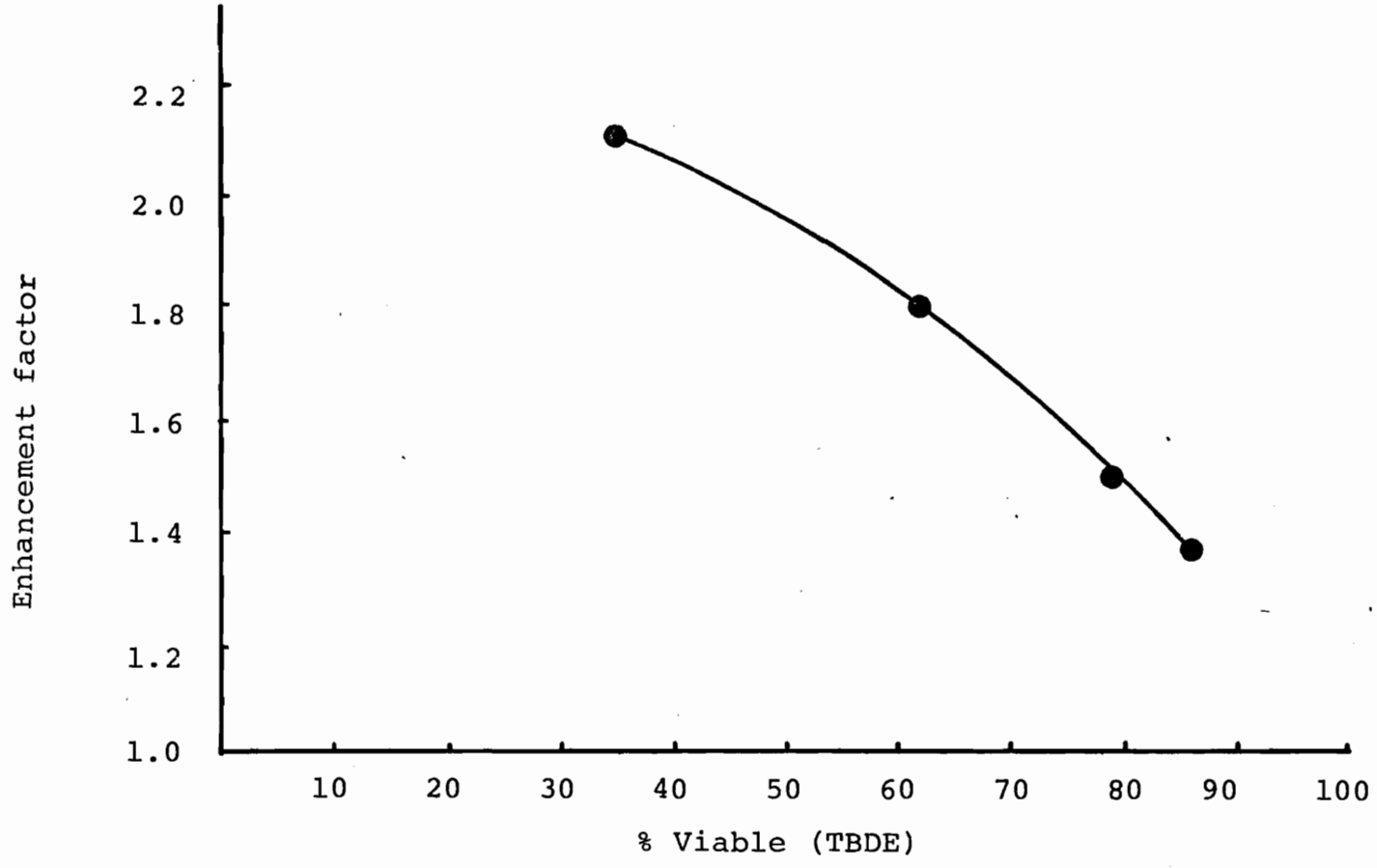


Figure 6. Comparison of NADPH-Norbenzphetamine Stimulation of Cell Respiration with Trypan Blue Dye Exclusion (TBDE)

With a new group of cells, 10 µl of 100 mM norbenzphetamine were added and the rate noted, followed by 10 µl of 60 mM NADPH. This procedure was also performed in duplicate. A norbenzphetamine concentration well above the substrate optimum for MI complex formation was used to ensure that the norbenzphetamine metabolism did not become self-limited.

The "enhancement factor" of norbenzphetamine could then be calculated as follows:

$$\frac{R_{NADPH+NB+ER} - R_{NADPH+ER}}{R_{NB+ER} - R_{ER}} = \text{Enhancement Factor}$$

where $R_{NADPH+NB+ER}$ is the mean rate of consumption of oxygen after addition of both NADPH and norbenzphetamine

$R_{NADPH+ER}$ is the mean rate after addition of just NADPH

R_{NB+ER} is the mean rate after addition of just norbenzphetamine

and R_{ER} is the mean endogenous rate.

Storage Conditions

Several experiments were performed to determine the storage conditions giving optimum sustained viability. Attention was directed primarily to temperature and oxygenation of the cell suspension.

Temperature

Cells stored at room temperature and above soon developed "balloon-like" projections of cytoplasm (Plate 2). This process occurred more quickly at 37° than 25°. No such projections appeared when cells were stored at 4°. While these projections were not clearly associated with an increase in LDHA or decrease in TBDE, cells showing them were considered compromised.

Gassing of the Cell Suspension

Using LDHA as the viability criterion, cells bubbled with 100% oxygen deteriorated much more rapidly than ungassed controls, both at 4° and 25°. Cells ungassed at 4° had the slowest rate of deterioration, cells gassed at 25° the fastest.

It was unclear from this experiment whether it was the mechanical aggitation of the bubbling, a change in pH, or excessive oxygen tension that caused the rapid deterioration in the oxygen-gassed cells. The experiment was repeated with compressed air and again with nitrogen. In neither case could a pH shift account for the increased mortality. It was doubtful that compressed air gassing, and impossible that nitrogen gassing, would lead to an excessive oxygen tension. It was concluded that the aggitation produced by bubbling presented a serious threat to the viability of the cell preparation in storage.

While, perhaps, bubbling was not necessary, and blowing the gas over a shallow suspension may allow for sufficient gas exchange (Krebs, 1976), the need to provide oxygen for cells stored at 4° was questioned. Cells stored ungassed at 4° for two hours showed only a 10% increase in number of cells taking up trypan blue. Fry et al (1976) have reported that viability was maintained for at least 4 hours when their cell suspensions were stored in Hanks BSS at 4°.

The NADPH/$NADP^+$ ratio for several cell suspensions was determined according to the method of Klingenberg (1974), both at the time of isolation and after two hours of storage at 4°. There was some concern that despite a high TBDE viability, cells deprived of oxygen in storage would deplete their supplies of reduced pyridine nucleotides, making them unsuitable for drug metabolism studies.

Both the NADP(H) total concentration and the percent in the reduced form (Table 5) compared favorably with results published earlier (Moldeus et al., 1974). While the NADP(H) concentration after two hours appears higher than that immediately after isolation, it should be noted that two observations were made at two hours compared with six at 0 hours. The variability of samples would probably account for this

TABLE 5

NADP(H) Concentration and Percent Reduced in Hepatocytes Freshly Isolated and Those Stored at 4° for Two Hours

	NADP(H) Concentration nmoles/10^6 cells	% Reduced
Freshly isolated[6] (0 hr.)	3.88 ± .50	89 ± 2
Stored for 2 hr.[2]	4.58 ± 1.28	88 ± 1

Numerical superscripts refer to the number of samples.

paradoxical increase. The percent of the total triphosphopyridine nucleotide in the reduced form remained constant during the two hours, indicating that reduced pyridine nucleotide stores are not depleted when the suspension stored at 4° is not gassed.

Spectrophotometric Examination of Isolated Cells

Cell Culture

While light absorbance can be measured in the intact hepatocyte, suspensions settle rather quickly in a cuvette, causing a diminishing signal and making spectrophotometric assays impossible to perform. In order to minimize the settling problem, attempts were made to culture hepatocytes on the walls of the cuvette. Since only viable cells will afix themselves to glass (or plastic), all cells measured would have passed at least this one criterion of cell quality.

Four dilutions of cells were prepared under sterile conditions with L-15 culture medium. The cell/medium suspension was placed in eight cuvettes, one plastic and one glass for each dilution. The cuvettes were sealed with Parafilm^{R} and placed on their sides in petri dishes. Air holes were punched in the Parafilm^{R} above the level of the suspension. The cuvettes were incubated at 37° for 24 hours.

There was very little cell adherence to the plastic cuvettes. The glass cuvettes proved much better, and each was rinsed with saline to remove the dead cells.

Two of the cuvettes were filled with CO-gassed MHBSS and placed in an Aminco DW-2 spectrophotometer. With the instrument in the split beam mode, the cuvettes were balanced at 500 nm. The sample cuvette was then reduced with sodium dithionite and a difference spectrum recorded. The signal from the monolayer of cells was too weak for the cytochrome P-450 to be measured. Doubling of the signal by culturing cells on both inside walls of the cuvette exposed to the light beam would still not produce sufficient signal for accurate measurement of cytochrome P-450 content. Since the absorbance typically encountered in MI complex measurement is less than that for cytochrome P-450 measurement, cell culture did not provide a satisfactory solution to the settling problem.

Magnetic Stirrer

Another alternative was to use a magnetic stirrer to keep the cells from settling. A specialized accessory for the Aminco DW-2 was obtained which permits magnetic stirring of the cuvettes with a minimum of magnetic disturbance to the photomultiplier.

The amount of disturbance the magnetic stirrer would produce with the cells was unknown. Samples of cells were stirred at the temperature to be used for assays (25°) and periodically checked for their TBDE and LDHA activity (Table 5). Unstirred controls were used to differentiate the effects of the magnetic stirrer from temperature effects. Cell suspensions, stirred and unstirred, showed the balloon-like projections described earlier at 10 minutes. While neither group of cells showed substantial decreases in TBDE viability, LDHA results indicated that damage was taking place in both groups, particularly the stirred suspension.

In subsequent experiments and assays, efforts were made to minimize the time the suspensions were exposed to magnetic stirring. Most assays required less than five minutes to perform.

Measurement of Cytochrome P-450 and MI Complex Formation

Cytochrome P-450 content was measured for both microsomal and hepatocyte suspensions in a manner similar to that described previously (Omura and Sato, 1964) assuming an extinction coefficient of 91 $mM^{-1}cm^{-1}$. Microsomal suspensions were measured at a concentration of 2.0 mg microsomal protein/ml. Hepatocyte suspensions were measured at 2×10^6 cells/ml. The method of

Buening and Franklin (1976) was used to ascertain the amount of cytochrome P-450 bound as the complex in both microsomes and isolated hepatocytes.

The maximum rate of MI complex formation in microsomes was determined according to the method of Franklin (1973). Measurement of MI complex formation in hepatocyte suspensions was performed in an analogous fashion except that NADPH addition was not required to initiate the reaction. Hepatocytes were diluted to a concentration of 2×10^6 cells/ml with oxygenated MHBSS just prior to assay.

Measurement of Complex Stability in Microsomes and Hepatocytes

Complex stability in microsomes from phenobarbital-pretreated rats was measured by first making the complex at 25° using the optimum substrate concentration of each compound (330 μM for N-hydroxyamphetamine). In some instances, the system was intentionally NADPH-limited with only 200 μM NADPH added. In other cases, NADPH was not limited and 400 μM was added and an NADPH regenerating system consisting of 7 mM isocitric acid (Type 1, Sigma Chemical Co., St. Louis, Missouri) and 0.2 units/ml isocitrate dehydrogenase (Sigma Chemical Co., St. Louis, Missouri) was included with the buffer. Buffer in all cases was oxygen-saturated 50 mM Tris-chloride buffer

(pH 7.4) containing 150 mM KCl and 10 mM $MgCl_2$. Microsomal concentration was 2.0 mg protein/ml. The MI complex was measured as described in "Measurement of Cytochrome P-450 and MI Complex Formation," above.

The rates of decay of MI complex in microsomes were followed at 10°, 15°, 20°, 25°, and 30° in NADPH-limited systems. When complex formation was complete, the temperature in the water-jacketed cuvette was rapidly changed utilizing a series of water baths. Transition time was usually less than 200 seconds. Temperature variations were $\pm$ 1°.

Measurement of MI complex stability in hepatocytes followed procedures very similar to those used for microsomes with the following exceptions: NADPH was not added nor was an NADPH regenerating system included in the buffer, the buffer used was MHBSS, and the hepatocyte concentration was 2.0×10^6 cells/ml.

RESULTS

The Formation of MI Complexes In Vitro

Hepatocyte suspensions from phenobarbital-pretreated animals were examined for their ability to form MI complexes (Table 6), and compared with results obtained previously from microsomal suspensions from similarly treated animals (James and Franklin, 1975b). With N-hydroxyamphetamine the rates of MI complex formation increased with substrate concentration with no diminished activity at the highest concentrations examined in both microsomes and hepatocytes. Thus, a substrate optimum for N-hydroxyamphetamine, if any, could not be determined accurately in either system. Norbenzphetamine and SKF 525-A produced distinct substrate optima which were substantially lower in hepatocytes as compared to microsomes. d-Amphetamine, which had the lowest rate of MI complex formation of the compounds examined in microsomal suspensions, did not produce a detectable rate of MI complex formation in hepatocytes with a similar substrate concentration range (3-1000 μM). In all cases, the maximum rates of MI complex formation were lower in hepatocytes

TABLE 6

The Formation of Cytochrome P-450 Metabolic Intermediate Complexes in Microsomes and Hepatocytes from Phenobarbital-Pretreated Rats

	MICROSOMES		HEPATOCYTES	
Substrate	Substrate Optimum	Maximum Rate[a,c]	Substrate Optimum	Maximum Rate[a]
	(μM)		(μM)	
N-hydroxyamphetamine	>330[b]	128.4 ± 9.0[8]	>330[f]	85.2 ± 7.9[2]
Norbenzphetamine	100[c]	11.96 ± .88[13]	9 ± 2[4]	1.86 ± .42[3]
SKF 525-A	33[d]	3.92 ± .21[12]	13 ± 2[3]	1.88 ± .41[3]
d-Amphetamine	>3300[e]	2.25 ± .13[13]	n.d.[2]	n.d.[2]

All numerical superscripts indicate the number of samples:

[a] Δ absorbance/μmole cytochrome P-450/minute

[b] from Franklin, 1974d; Km = 30 μM

[c] from James and Franklin, 1975b

[d] from Franklin, 1974a

[e] from Franklin, 1974c

[f] Km = 22 μM

n.d. indicates that no rate of MI complex could be detected

All values ± S.E.M.

than microsomes when compared on the basis of specific activity (Δ absorbance/μmole cytochrome P-450/minute).

A comparison of in vitro MI complex formation in hepatocytes derived from untreated and phenobarbital-pretreated rats appears in Table 7. Hepatocytes from untreated rats, as well as those from phenobarbital-pretreated animals, did not show a discernable substrate optimum for N-hydroxyamphetamine in the dose range examined (3-1000 μM). Hepatocytes from untreated animals did not produce detectable rates of MI complex formation with either SKF 525-A or d-amphetamine with the concentration of substrate employed (3-480 μM for SKF 525-A; 3-1000 μM for d-amphetamine).

Phenobarbital induction did not change the optimum substrate concentration for MI complex formation for norbenzphetamine in hepatocytes, nor does induction change the optimum in microsomes (Franklin, 1974c). Where MI complex formation occurred, phenobarbital induction increased the maximum activity approximately three- to six-fold. The ability of phenobarbital pretreatment to increase the rates of MI complex formation in microsomes is more dramatic, and increases of six-fold for N-hydroxyamphetamine (Franklin, 1974d) and 30-fold for norbenzphetamine (Franklin, 1974c) have been reported.

TABLE 7

The Formation of Cytochrome P-450 Metabolic Intermediate Complexes in Hepatocytes Derived from Untreated and Phenobarbital-Pretreated Rats

Substrate	Untreated		Phenobarbital-pretreated[a]	
	Substrate Optimum	Maximum Rate[b]	Substrate Optimum	Maximum Rate[b]
	(μM)		(μM)	
N-hydroxyamphetamine	>330[3]	.0177 ± .0027[3]	>330[3]	.1153 ± .0029[3]
Norbenzphetamine	10 ± 0[2]	.0015 ± .0001[2]	9 ± 2[4]	.0041 ± .0014[4]
SKF 525-A	n.d.[2]	n.d.[2]	13 ± 2[3]	.0030 ± .0010[3]
d-Amphetamine	n.d.[3]	n.d.[3]	n.d.[2]	n.d.[2]

[a] 80 mg/kg/day for four days

[b] Δ absorbance/2 x 10^6 cells/min

All values ± S.E.M.

Numerical superscripts indicate number of samples examined.

n.d. indicates that no rate of MI complex could be detected with the substrate concentrations employed.

Stability of MI Complexes Formed In Vitro in Microsomes and Hepatocytes

In NADPH-limited microsomal systems, MI complexes were found to be unstable. When the NADPH supply was exhausted the complex would decay, as indicated by a diminishing 455-490 nm absorbance (Figure 7). Addition of more NADPH resulted in new complex formation. If an NADPH regenerating system was included (7 mM isocitrate; 0.2 units/ml isocitrate dehydrogenase), the absorbance would increase to a maximum without diminishing in the time period examined (30 minutes).

The rate of decay of the MI complex formed from optimum substrate concentrations in NADPH-limited systems was found to be temperature-dependent (Figures 8, 9, 10). The rate of decay decreased with temperature, and the norbenzphetamine MI complex appeared to be stable within the time measured at 10°. The SKF 525-A MI complex appeared to stabilize at a higher temperature, 15°. The pattern of stability of N-hydroxyamphetamine was somewhat different than that for norbenzphetamine and SKF 525-A. The complex persisted at an apparent maximum for several minutes before decaying. At the lowest temperature examined, 10°, the MI complex from N-hydroxyamphetamine still showed a measureable rate of decay. Slopes of the

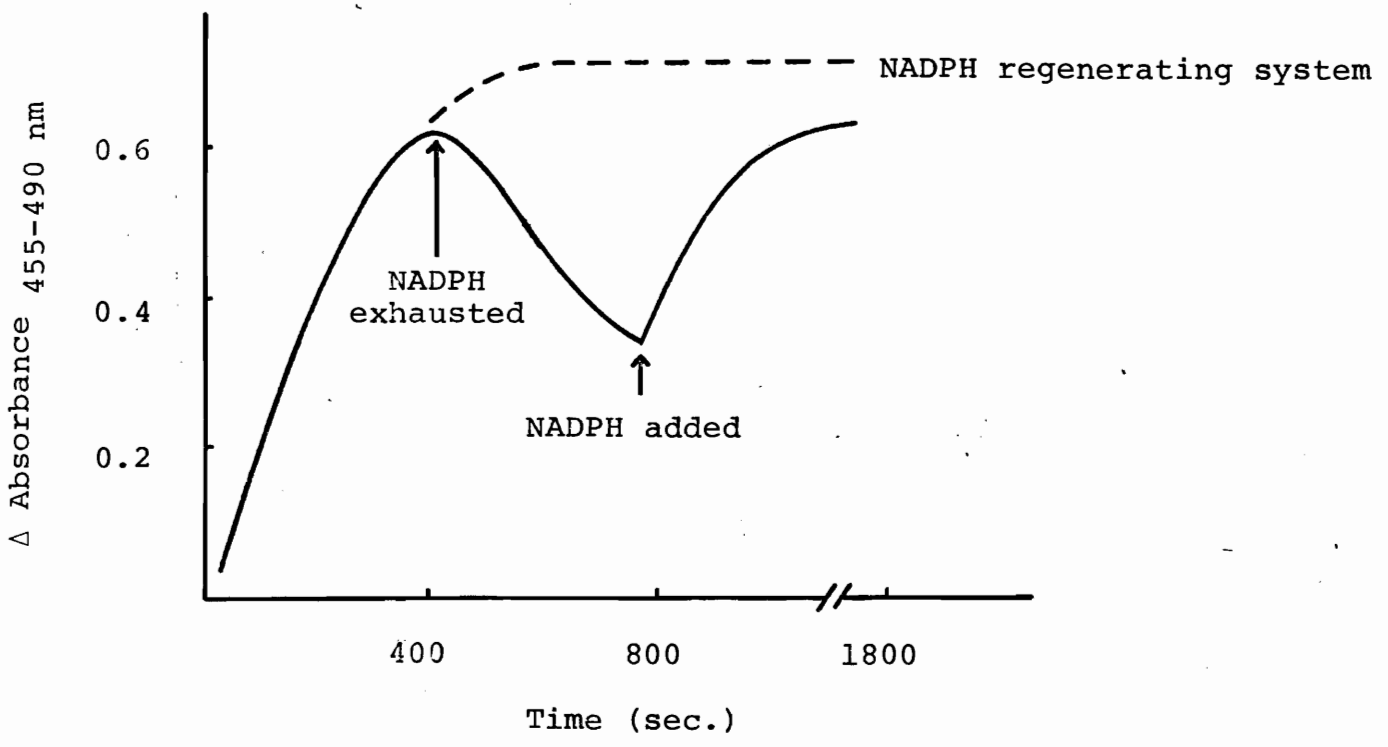
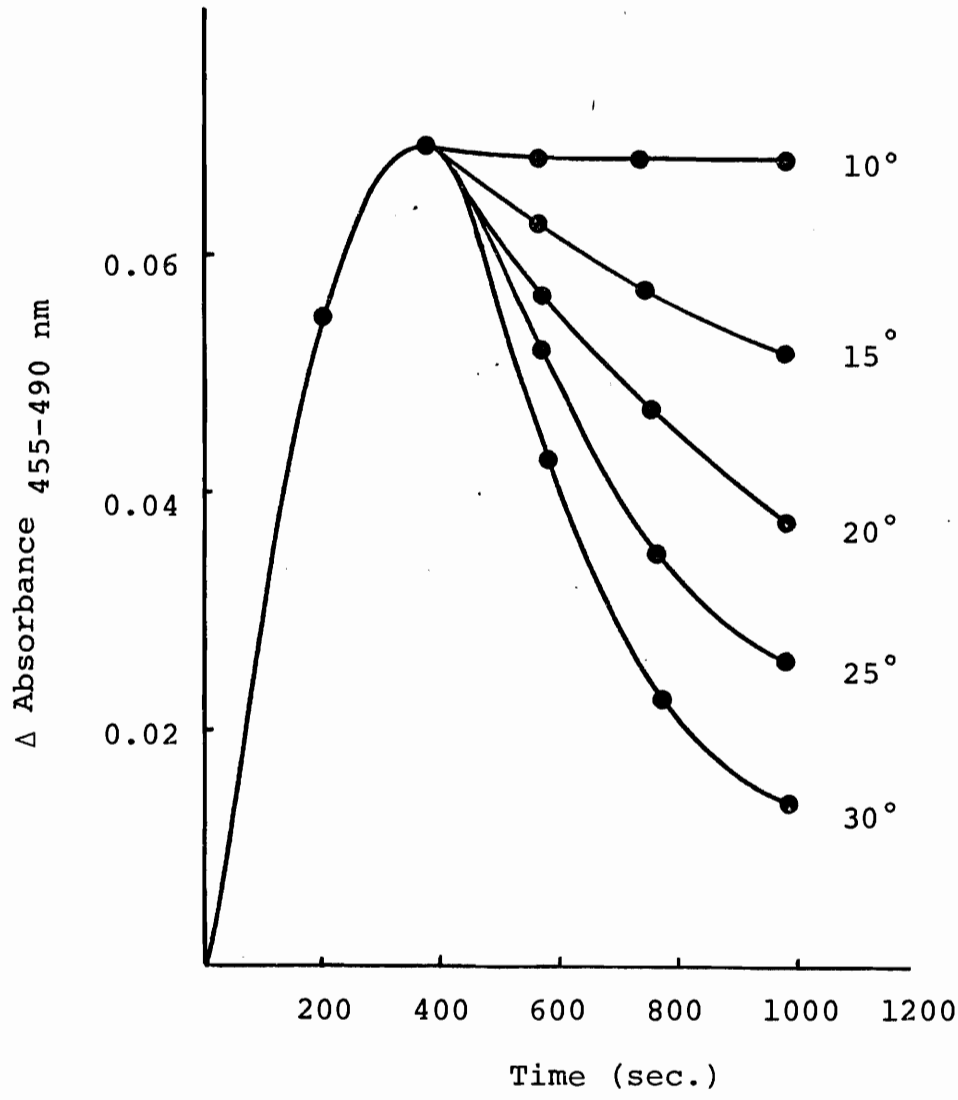
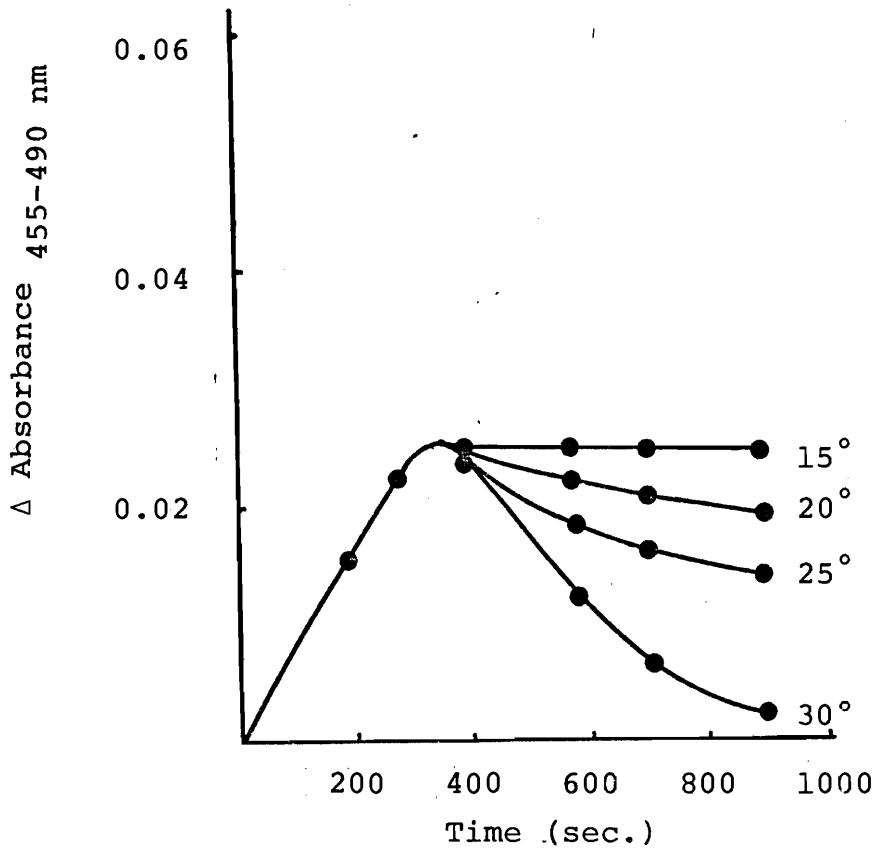


Figure 7. Effects of NADPH on Complex Stability



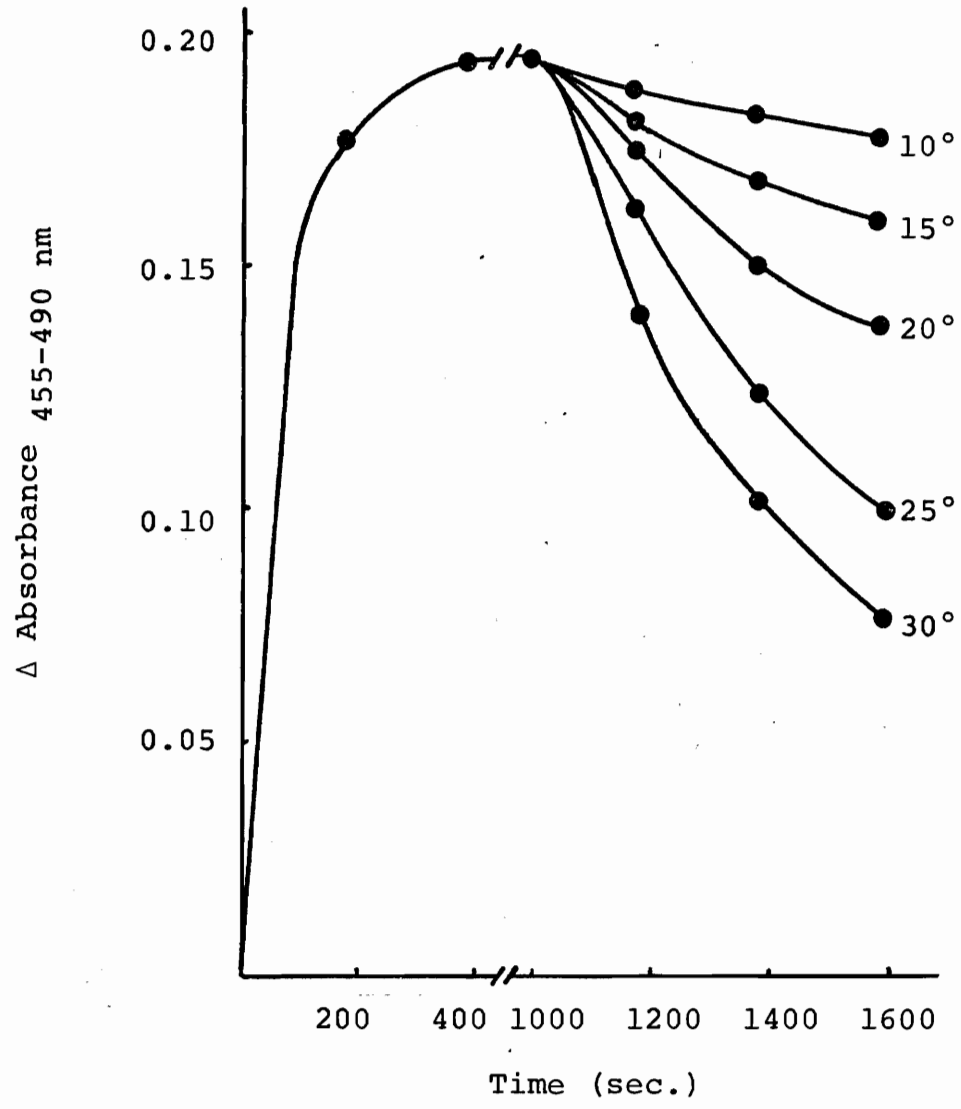
Points represent the means of two or more observations

Figure 8. The Effects of Temperature on the Rate of Decay of Complex Formed from Norbenzphetamine in an NADPH-Limited Microsomal System.



Points represent the means of two or more observations

Figure 9. The Effects of Temperature on the Rate of Decay of Complex Formed from SKF 525-A in an NADPH-Limited Microsomal System



Points represent the means of two or more observations

Figure 10. The Effects of Temperature on the Rate of Decay of Complex Formed from N-Hydroxyamphetamine in an NADPH-Limited Microsomal System

decay curves did not follow zero, first, or second order kinetics. Because of the mixed-order nature of the curves, values for slopes were not calculated.

The norbenzphetamine MI complex took much longer to reach an absorbance maximum in hepatocytes (Figure 11) as compared to microsomes. The MI complex was stable for about 15 minutes at 25° after reaching maximum (30 minutes after drug addition), and then began to decline.

MI Complex Formation In Situ as Measured in Cells and Microsomes Isolated from Drug-Treated Animals

Microsomes derived from phenobarbital-pretreated rats given 100 mg/kg (i.p.) SKF 525-A one hour prior to sacrifice showed up to 21% of the cytochrome P-450 bound as the MI complex (Table 8). This is a lower value than reported earlier (maximum of 41%) by Buening and Franklin (1976). Cytochrome P-450 concentrations from these experiments compared to those of Buening and Franklin (1976) (3.35 nmole/mg protein) were much lower, however. Hepatocytes isolated from identically treated animals showed similar amounts of MI complex to that seen in microsomes but with a greater variation.

Phenobarbital-pretreated rats given norbenzphetamine in doses ranging from 10 to 100 mg/kg (i.p.)

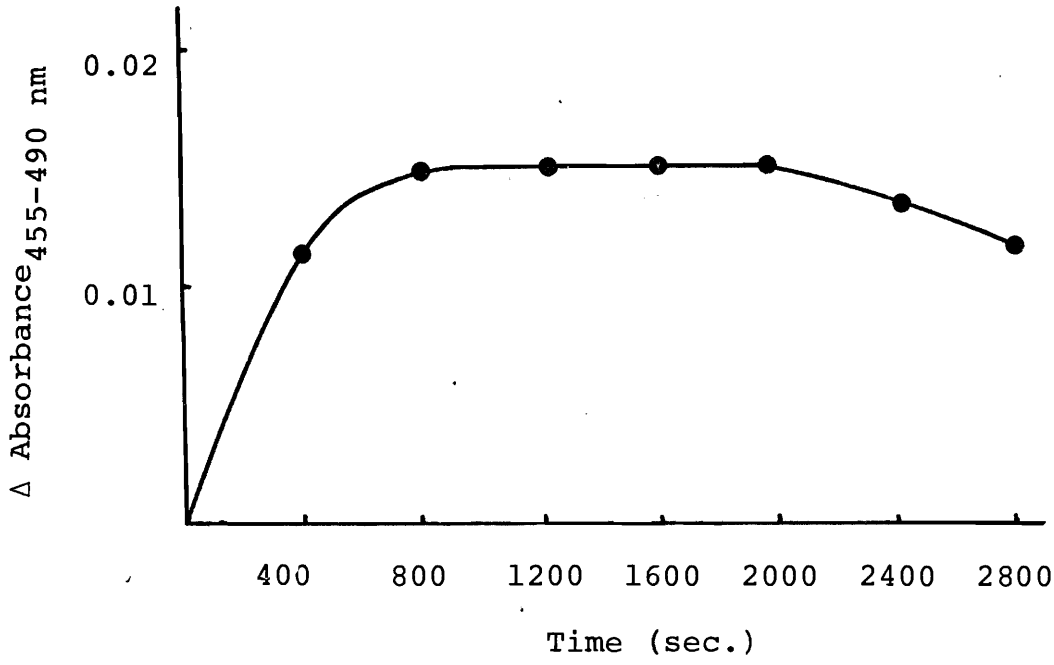


Figure 11. The Stability of Norbenzphetamine Complex in Hepatocytes at 25°

TABLE 8

MI Complex in Microsomes and Hepatocytes Derived from SKF 525-A Treated[a] Rats[b]

Microsomes		Hepatocytes	
Cytochrome P-450[c]	% Cyt. P-450 as complex	Cytochrome P-450[d]	% Cyt. P-450 as complex
1.27	16	--	--
1.40	21	0.82	33
1.45	0	--	--
1.85	19	0.86	12
2.31	18	0.72	0
1.66 ± .19[3]	15 ± 4[3]	0.80 ± .04[e]	15 ± 10[e]

[a] 100 mg/kg SKF 525-A, i.p., one hr prior to sacrifice

[b] Phenobarbital pretreated, 80 mg/kg/day for 4 days

[c] nmole/mg protein

[d] nmole/10^6 cells

[e] mean ± S.E.M.

yielded microsomes containing no more than 2% of the cytochrome P-450 bound as the MI complex. Hepatocytes from norbenzphetamine-treated rats possessed a maximum of 9% cytochrome P-450 as the MI complex.

DISCUSSION

Isolated hepatocyte suspensions have several advantages in the study of some drug metabolism problems. They retain the complex biochemical mechanisms of the intact cell while offering the versatility and control of an *in vitro* model system. Since hepatocyte suspensions can be examined directly by spectrophotometry, they are particularly useful for the study of rapid reactions associated with absorbance changes such as cytochrome P-450 MI complex formation.

In many respects, the isolated hepatocyte suspension is a model system intermediate between subcellular fractions (microsomes) and intact tissue (isolated perfused liver). However, it is unique among the model systems in that the cell need not be disrupted and the endoplasmic reticulum recovered before the results of reactions are quantitated spectrophotometrically. These features made the isolated hepatocyte suspension particularly useful in this study.

In vitro MI complex formation in hepatocytes proved very similar to that in microsomes. The substrate optimum phenomenon is not unique to microsomes

but is evident in the intact cell as well. With the exception of MI complex formation with d-amphetamine, differences between the two systems appear to be quantitative rather than qualitative, with hepatocytes showing both lower substrate optima and specific activities. While no substrate optimum concentration could be determined for N-hydroxyamphetamine in either hepatocytes or microsomes, the Km values were comparable, 30 μM in microsomes (Franklin, 1974d) and 22 μM in hepatocytes.

The lower specific activity of MI complex formation in hepatocytes as compared with microsomes corresponds with observations of other investigators reporting a similar relationship for other mixed-function oxidation reactions (Henderson and Dewaide, 1969; Vadi et al., 1975). It is possible that mixed-function oxidation reactions are limited by the rate of substrate access to the endoplasmic reticulum in the intact cell, but apparent lower substrate optimum concentrations suggest that cells may, in fact, concentrate the substrate. The observations of Moldeus et al. (1974) on the $NADPH/NADP^+$ ratios in rats starved and fed, untreated and phenobarbital-pretreated, and their rates of metabolism of alprenolol suggest that, especially with phenobarbital-pretreated animals, rates of drug metabolism in intact hepatocytes may be NADPH-limited.

Comparisons of substrate optimum concentrations obtained in isolated hepatocytes with concentration ranges used in isolated perfused liver studies (Table 2) and doses given whole animals cannot resolve whether or not the doses and concentrations used were below, above, or within the optimum range. Previous experiments with whole animals did not attempt to establish plasma or tissue concentrations of the drug, and while total concentrations of drug in the isolated perfused liver systems are known, the free drug concentration (i.e., not bound to albumin, glass, etc.) is not known. Further experimentation will be required to determine the role of the substrate optimum in the dissimilar in vitro and in vivo MI complex observations. The isolated perfused liver system should be re-examined with known concentrations of free drug near the respective substrate optimum concentrations for isolated hepatocytes. The assumption will have to be made for these experiments that the subcellular distribution of the drug is the same whether the cell is isolated or situated in intact tissue.

The stability of MI complexes formed in vitro in microsomes is dependent upon a continued supply of reducing equivalents from NADPH. MI complexes in both microsomes and hepatocytes are stable in the presence of the reducing agent sodium dithionite but unstable

when oxidized by potassium ferricyanide. The role of NADPH in MI complex stability could involve maintaining the cytochrome P-450 MI complex in a reduced or semi-reduced state, in which case depletion of the NADPH and subsequent oxidation of cytochrome P-450 would break the MI complex. On the other hand, the MI complex may be inherently unstable and the presence of NADPH allows the formation of replacement MI complex from remaining substrate.

The two mechanisms could be distinguished by depleting the microsomal system of substrate so that no new complex could be formed. In practice, this proves very difficult. The slow decay of MI complex 15 minutes after reaching maximum (30 minutes after drug addition) in hepatocytes, which in theory will maintain reduced triphosphopyridine nucleotide, may reflect the exhaustion of substrate. This decay may also be the result of cell damage from stirring or the inability to sustain NADPH supplies. In microsomal suspensions containing an NADPH regenerating system decay does not appear in the same period of time. The problem is further complicated in that the metabolites of many MI complex-forming substrates are themselves capable of forming an MI complex.

Hepatocytes isolated from drug-treated animals appear to maintain supplies of reduced pyridine

nucleotides during cell preparation. Thus, MI complex formed *in situ* may have continuous availability of NADPH during cell isolation. The subcellular distribution of the reduced pyridine nucleotides is unknown, and the 90%-reduced values may not apply to the NADP(H) at the endoplasmic reticulum. If it is assumed that adequate NADPH is present during cell preparation, then a redox role for NADPH in complex stability would be discounted, as the amounts of complex found in these hepatocytes are quite low when compared to *in vitro* results for SKF 525-A and norbenzphetamine.

MI complexes formed *in vitro* from SKF 525-A, norbenzphetamine, and N-hydroxyamphetamine all showed temperature-dependent decay rates. There were differences in the effects of temperature on the decay rates among the compounds. At the lower temperatures, MI complex from SKF 525-A was more stable than that from norbenzphetamine which, in turn, was more stable than N-hydroxyamphetamine MI complex. This order of stability at low temperatures parallels the amounts of complex which appear in both hepatocytes and microsomes from drug-treated animals.

The cause of the temperature-dependent decay is not known. It may represent a simple dissociation or an enzymatic reaction. The mixed-order nature of the

decay rate makes interpretation difficult, and the decay reaction could not be analyzed for the breaks in Arrhenius plots shown for other reactions in microsomes such as the dealkylation of 7-ethoxycoumarin (Duppel and Ullrich, 1976), glucose-6-phosphatase activity, and UDP-glucuronyltransferase activity (Eletr et al., 1973).

It is not clear how the temperature-dependent decay can account for the observed amounts of complex. Significant amounts of SKF 525-A MI complex appear both in microsomes prepared at 4° and hepatocytes prepared at 37°. Almost no complex is detected from either microsomes or hepatocytes from norbenzphetamine-pretreated animals.

There are a number of possible explanations for these observations. It is possible that very low amounts of norbenzphetamine and N-hydroxyamphetamine MI complex are formed in vivo as compared to in vitro. However, such is not the case with complex from SKF 525-A, and it seems unlikely that the process of cell isolation would selectively and substantially enhance MI complex-forming ability with the two amphetamine derivatives over what the cell possesses in situ.

Amounts of MI complex comparable to those formed in vitro in microsomes may form in vivo, but decay

rapidly. Thus, the amounts of MI complex assayed may not be too different from the amounts present at sacrifice. The timing of sacrifice after the dose would, therefore, become of critical importance. A thorough examination of the timecourse of MI complex formation after administration of the drug could prove useful in exploring this possibility.

The seeming agreement between hepatocytes and microsomes from drug-treated animals may be the result of two different destructive processes--temperature-related decay in the case of hepatocytes and the mechanical disruption Franklin observed (unpublished results) in the case of microsome preparation. A tightly-bound complex, perhaps like that from SKF 525-A, would be expected to resist both forces better than a weaker complex.

The results of the temperature studies have indicated the importance of keeping the sample cold when attempting to determine in vitro the amount of in vivo MI complex formation. A differential effect of temperature on the decay rates of MI complexes from different substrates has been demonstrated. The effects of mechanical disruption (e.g., during microsome preparation) should also be studied for a differential effect.

The presence of a decay rate implies that the amount of MI complex seen at any given time is the result of two rates, the rate of formation and the rate of decay. Low amounts of MI complex may be due to a high rate of decay relative to the rate of formation, and MI complex stability would be the result of a steady-state balance between formation and decay. This important concept means that the amount of cytochrome P-450 susceptible to MI complex formation is not the only limiting factor in the amounts of MI complex which can be formed.

It is possible that sufficient substrate remains bound to allow for the formation of new complex during hepatocyte preparation. A difference in binding for SKF 525-A, norbenzphetamine, and N-hydroxyamphetamine in such a case would support this explanation but has not been demonstrated. Microsomes, while prepared at temperatures yielding apparent stability of the MI complex, would not form new complex for reasons of low temperature and the absence of NADPH.

Determining the presence of bound residual substrate in hepatocytes during cell isolation would be a difficult task. While sophisticated analytical techniques may reveal the presence of substrate, they cannot tell if it is bound in a position to be available

for MI complex formation. Depletion of NADPH in the hepatocytes during preparation would stop potential new MI complex formation, but would be expected to produce other complicating effects within the cell. The demonstration of substrate binding in microsomes during preparation would provide some supporting evidence, and could be examined by adding NADPH to freshly prepared microsomes from drug-treated animals and monitoring for 455-490 nm absorbance changes.

It is apparent that more research will be required to ultimately resolve the differences between observations of MI complex formation in microsomes, isolated perfused livers, and whole animals. The problem remains an important issue, however, because of its implications on the prediction of *in vivo* (and clinical) inhibition of drug metabolism. The isolated hepatocyte suspension is of proven utility in this investigation, and experiments with this model, as well as others, will, hopefully, elucidate the mechanisms underlying past experimental observations.

Addendum: During the proofing of this manuscript, a research paper of relevance to this work appeared in the literature (Hirata et al., 1977). These workers

examined MI complex formation in vitro from norbenzphetamine and N-hydroxyamphetamine in microsomes and isolated hepatocytes.

Hirata et al. examined the relationship of gluthathione to MI complex formation in both microsomes and hepatocytes. They found that glutathione inhibited the rate of MI complex formation from norbenzphetamine in microsomes and that isolated hepatocytes depleted of glutathione formed MI complex much better than hepatocytes containing normal concentrations of glutathione. It was also reported that glutathione increased the rates of decay of the MI complexes. No specific mechanisms for these effects of glutathione were presented.

During their investigation, they found that both norbenzphetamine and N-hydroxyamphetamine formed MI complexes in hepatocytes and microsomes from phenobarbital-pretreated rats. They noted that the rate and extent of formation of MI complex from norbenzphetamine were substantially lower in hepatocytes as compared to microsomes. MI complex with d-amphetamine was not observed in hepatocytes. These results are in agreement, at least qualitatively, with data derived in this thesis. Because of the manner in which the results of Hirata et al. are presented, precise comparisons with this thesis are difficult, but

it appears that the rate and amount of MI complex formation from norbenzphetamine observed by these investigators was low. This can be explained by the fact that Hirata et al. used norbenzphetamine concentrations ($\geq$ 100 μM) above the substrate optimum for norbenzphetamine in isolated hepatocytes.

Norbenzphetamine was found to be an uncoupler of oxidative phosphorylation. This observation increases the complexity of interpretation of results obtained here concerning the NADPH-norbenzphetamine stimulation of cell respiration as a viability test.

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